Genetic Diversity and New Lineages of Dengue Virus Serotypes 3 and 4 in Returning Travelers, Germany, 2006–2015

Sami Shihada, Petra Emmerich, Corinna Thomé-Bolduan, Stephanie Jansen, Stephan Günther, Christina Frank, Jonas Schmidt-Chanasit, Daniel Cadar

During 2006–2015, we analyzed 70 dengue virus (DENV) strains isolated from febrile travelers returning to Germany. High genetic diversity, including multiple co-circulating DENV lineages and emerging new lineages of DENV-3 and DENV-4, was demonstrated. Our passive surveillance system based on returning travelers yielded substantial information on DENV diversity.

Although dengue virus (DENV) infects ≈390 million persons annually and one third of the world's population is at risk for infection, there is no effective vaccine or specific antiviral therapy for infection with DENV (*1*). Dengue is a rapidly spreading mosquito-borne viral disease and the most frequent cause of febrile illness among international travelers returning from DENV-endemic tropical areas, such as Southeast Asia, the western Pacific region, and the Americas (*2*,*3*). Viremic travelers have the potential to introduce DENV into DENV-free or nonendemic areas where competent mosquito vectors are present (*4*). Reintroduction of DENV in regions that had been considered free of the disease for many years has also been observed (*5*–*7*).

Phylogenetic analysis has elucidated the origins, epidemiology, and forces that shape DENV molecular evolution in nature (*8*). For example, according to official German air travel statistics reports, 4,855,763 air trips were taken in 2011 from Germany to countries listed as DENV-endemic areas by the World Health Organization; 10%–20% each of these trips were made to India, Thailand, and Brazil; and 5%–10% each flew to Singapore, Mexico, and the Dominican Republic (*9*). We determined the genetic relatedness and molecular epidemiology of DENV isolates from travelers returning to Germany during 2006–2015.

Author affiliations: Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (S. Shihada, P. Emmerich, C. Thomé-Bolduan, S. Jansen, S. Günther, J. Schmidt-Chanasit, D. Cadar); Robert Koch Institute, Berlin, Germany (C. Frank); German Centre for Infection Research, Hamburg (J. Schmidt-Chanasit)

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The Study

During 2006–2015, we analyzed 15,876 acute-phase serum samples from patients with suspected DENV infection; the samples had been submitted to the World Health Organization Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research for diagnostic testing. We tested all samples by using DENV type-specific real-time reverse transcription PCR (rRT-PCR) (*10*) or an antigen-capture ELISA (Platelia Dengue NS1 Ag; Bio-Rad, Hercules, CA, USA) and in-house DENV IgG and IgM indirect immunofluorescence assays. rRT-PCR– and nonstructural protein 1–positive serum samples that tested negative for DENV IgG and IgM were spread onto Vero E6 cells and incubated for 7 days at 37°C; successful DENV isolation was identified by rRT-PCR. We extracted viral RNA from cell culture supernatants by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany).

We successfully isolated 70 DENV strains originating from 20 countries (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/23/2/16-0751-Techapp1.pdf). We amplified the complete envelope glycoprotein (E) gene using DENV type–specific degenerate primers (online Technical Appendix). Sequence assembly, analysis, and multiple alignments were performed with Geneious version 7.1.8 (Biomatters, Auckland, New Zealand). All available complete envelope gene sequences of DENV serotypes 1–4 (DENV-1–4), except laboratory strains and potential recombinants, were retrieved from GenBank and compared with those sequenced in this study. The phylogenetic relationships and origin of the imported DENV isolates were analyzed by the maximum likelihood method in the RAxML program (*11*) with general time-reversible plus gamma distribution substitution model and a rapid bootstrap (100 replicates) procedure, and visualized in FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Most DENV infections were acquired in Thailand (35.7%), followed by Indonesia (12.8%), the Philippines (10%), and India (7.1%). The proportion of cases imported from other countries in Asia, Africa, and the Americas ranged between 1.4% and 4.3% (Figures 1, 2). Phylogenetic analysis revealed a high genetic diversity of DENV-1–4 in travelers from Germany, including co-circulation of multiple genetically diverse viral lineages that were closely related to those previously circulating in the Americas and Southeast Asia (mostly Thailand, Indonesia, and

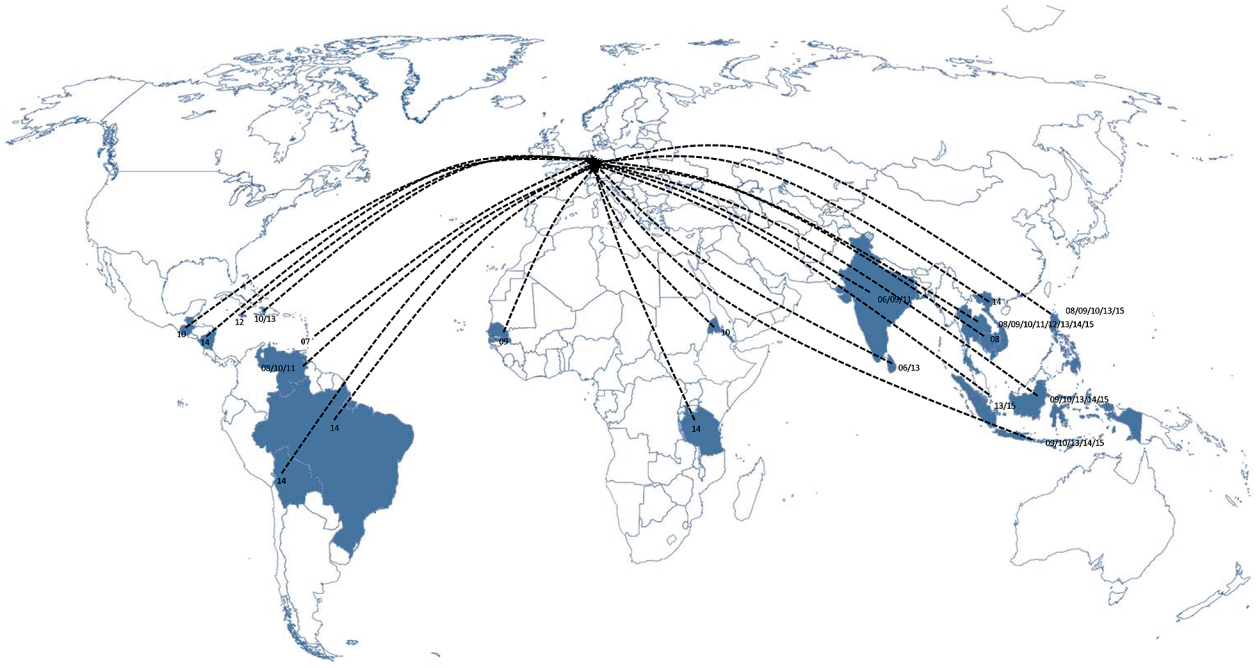

Figure 1. Geographic origin of dengue viruses isolated from travelers returning to Germany, 2006–2015.

the Philippines) (online Technical Appendix). DENV-1 was the predominant and most genetically diverse type, representing 33 sequences and clustering into 23 phylogenetically distinct lineages. Most of the isolates belonged to genotypes I and II, which are circulating in Southeast Asia (*12*). These genotypes represent dominant regional variants; only a few strains are closely related to viruses circulating in the Americas (genotype V, lineages 3–6) or to a genotype III virus in India. All but 3 DENV-2 isolates were the Cosmopolitan genotype, and the source population was limited mostly to countries in Southeast Asia (online Technical Appendix).

DENV-2 phylogeny provides evidence for 7 phylogenetically distinct introductions of the virus into travelers from Germany; the origins of these isolates were predominantly in Bali and the Philippines. DENV-3 sequences belonged to genotypes I–III; these isolates were closely related to lineages representing regional or local variants, except lineages 8 and 9 (online Technical Appendix).

A key phylogenetic pattern was the presence of a new lineage, L6 (online Technical Appendix), within DENV-3 phylogeny comprising closely related isolates sampled from German, Taiwanese, and Chinese travelers returning from Thailand and Laos during 2012–2014. A similar pattern of clustering to that for DENV-3 was observed for DENV-4 isolates. Thus, most DENV-4 from travelers were infected with genotype I and III strains closely related to lineages commonly sampled within Southeast Asia and to a genotype II isolate from Bolivia. A key phylogenetic pattern was the presence of a new lineage, L3, within DENV-4 phylogeny (online Technical Appendix) with sequences found in samples from patients tested during 2013–2015 with travel histories to Thailand, China, and the Philippines (lineage 3). Lineage 3 was the most recently circulating lineage detected in this study. This clustering is compatible with extensive viral traffic between Thailand, China, and the Philippines; Thailand, where most of the DENV infections were acquired, is thus a possible source of a virus population responsible for local or regional outbreaks.

Conclusions

Countries in Southeast Asia that are considered DENV hyperendemic are increasingly popular tourist destinations for residents of Germany; thus, German travelers to these countries are potentially exposed to multiple types and genotypes of DENV. A high prevalence of DENV infection has been reported in travelers returning from DENV-endemic areas, emphasizing the importance of international travelers as potential sources of imported disease or sentinels for local outbreaks in DENV-free or non–DENV-endemic areas (*2*,*3*). The relative risk of infection by country is difficult to calculate without attention to seasonal fluctuations in dengue fever incidence and travel patterns. Broadly speaking, though, among the top contributing countries mentioned, the comparative risk of infection with travel-associated DENV appears much higher in the Philippines and Indonesia (10% and 12.8% of the cases, compared with 1.7% and 2.8%, respectively, of travelers from Germany to DENV-endemic countries) than in Thailand (35.7% of the cases versus 15.0% of the

travelers) and the lowest in India (7.1% of the cases versus 18.5% of the travelers).

We investigated DENV diversity and origin of infection in travelers returning to Germany from DENV-endemic areas and identified a high genetic diversity of DENV genotypes and lineages. Notably, 2 of these lineages (DENV-3, genotype III, lineage 6 and DENV-4, genotype III, lineage 6) appear to have emerged very recently and are still responsible for local outbreaks in countries in Southeast Asia, thus reiterating the need to monitor the appearance and spread of novel lineages. Most investigated isolates were closely related to lineages known to have circulated in Thailand, the Philippines, and Indonesia, indicating that these countries serve as a major source of multiple DENV lineages (*12*). The observed high numbers of co-circulating lineages in the Thai, Indonesian, and Philippines source populations support the hypothesis of multiple geographic origins or extensive virus interchange among these countries (online Technical Appendix).

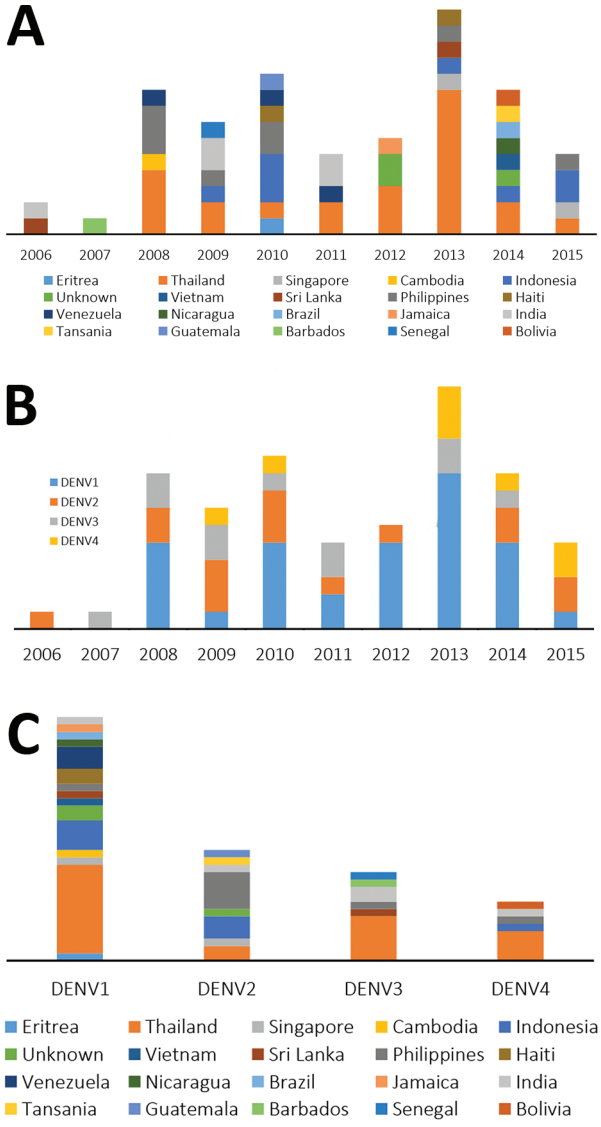

Figure 2. Year of introduction (A) and diversity (B and C) of dengue viruses isolated from travelers returning to Germany, 2006–2015.

Surveillance of symptomatic returned travelers can provide information on circulating DENV genotypes and lineages in heavily visited tourist areas and DENV-endemic regions. In Europe, the emergence of arboviruses should be particularly monitored because of the introduction and expansion of the DENV vector *Aedes albopictus* mosquito. In Germany, where recently introduced *Ae. albopictus* mosquitos have spread in the southwestern part of the country, international travelers and the presence of competent vectors could potentially facilitate seasonal local transmission of DENV (*7*,*13*,*14*). Our findings indicate a diverse array of imported DENV infections in travelers from Germany and emphasize the need for a continued surveillance of DENV infections in non–DENV-endemic regions as well as prompt and rapid serologic and molecular testing for DENV infection in febrile patients returning from DENV-endemic countries.

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Dr. Shihada is a physician at the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany. His research interest is characterization of human and zoonotic viruses.

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Address for correspondence: Jonas Schmidt-Chanasit, Bernhard Nocht, Institute for Tropical Medicine, World Health Organization Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany; email: jonassi@gmx.de

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Genetic Diversity and New Lineages of Dengue Virus Types 3 and 4 in Travelers Returning to Germany, 2006–2015

Technical Appendix

Technical Appendix Table 1. Primers used for the amplification and sequencing of complete envelope gene of dengue virus types

Primer name/serotype	Primer sequence, 5′→3′	Position and orientation*	Length, bp
PANENV-1F-DENV-1	ASA CRT GGG TGA CYT ATG GVA	557–577 F	1071
PANENV-1R-DENV-1	CAG TRT GCA TYG CTC CTT CYT	1607–1627 R	
PANENV-2F-DENV-1	CAG CYC ATG CDA ARA AVC AGG	1565–1585 F	858
PANENV-2R-DENV-1	TGT ATT GCT CTG TCC ARG TGT	2402–2422 R	
PANENV-1F-DENV-2	TCG CTC YTT CAA TGR CRA TGC	824–844 F	912
PANENV-1R-DENV-2	ATG ACA TTC CTT TRA GYT GT	1716–1735 R	
PANENV-2F-DENV-2	GGD TCY CAA GAD GGG GCY ATG	1600–1620 F	821
PANENV-2R-DENV-2	TAY TGY TCT GTC CAY GTR TGY	2400–2420 R	
PANENV-1F-DENV-3	CAC GYR CYC ARA CYT GGA TGT	656–676 F	656
PANENV-1R-DENV-3	ATT GCT CCY TCT TGI GAY CCA	1593–1613 R	
PANENV-2F-DENV-3	CCA TGG RCA TCA GGR GCI AYA	1489–1509 F	868
PANENV-2R-DENV-3	GTT CTT TMC CRT TCC ART TTA	2336–2356 R	
PANENV-1F-DENV-4	CCA TCY TAY GGA ATG CGI TGC	826–846 F	782
PANENV-1R-DENV-4	TCY TGA GAY CCR ARY ACT GTC	1597–1607 R	
PANENV-2F-DENV-4	CCT CAT GCC AAG AGA CAG GAT	1564–1584 F	784
PANENV2R-DENV-4	TCC ATG ACA CCA CAC ARC CC	2328–2347 R	

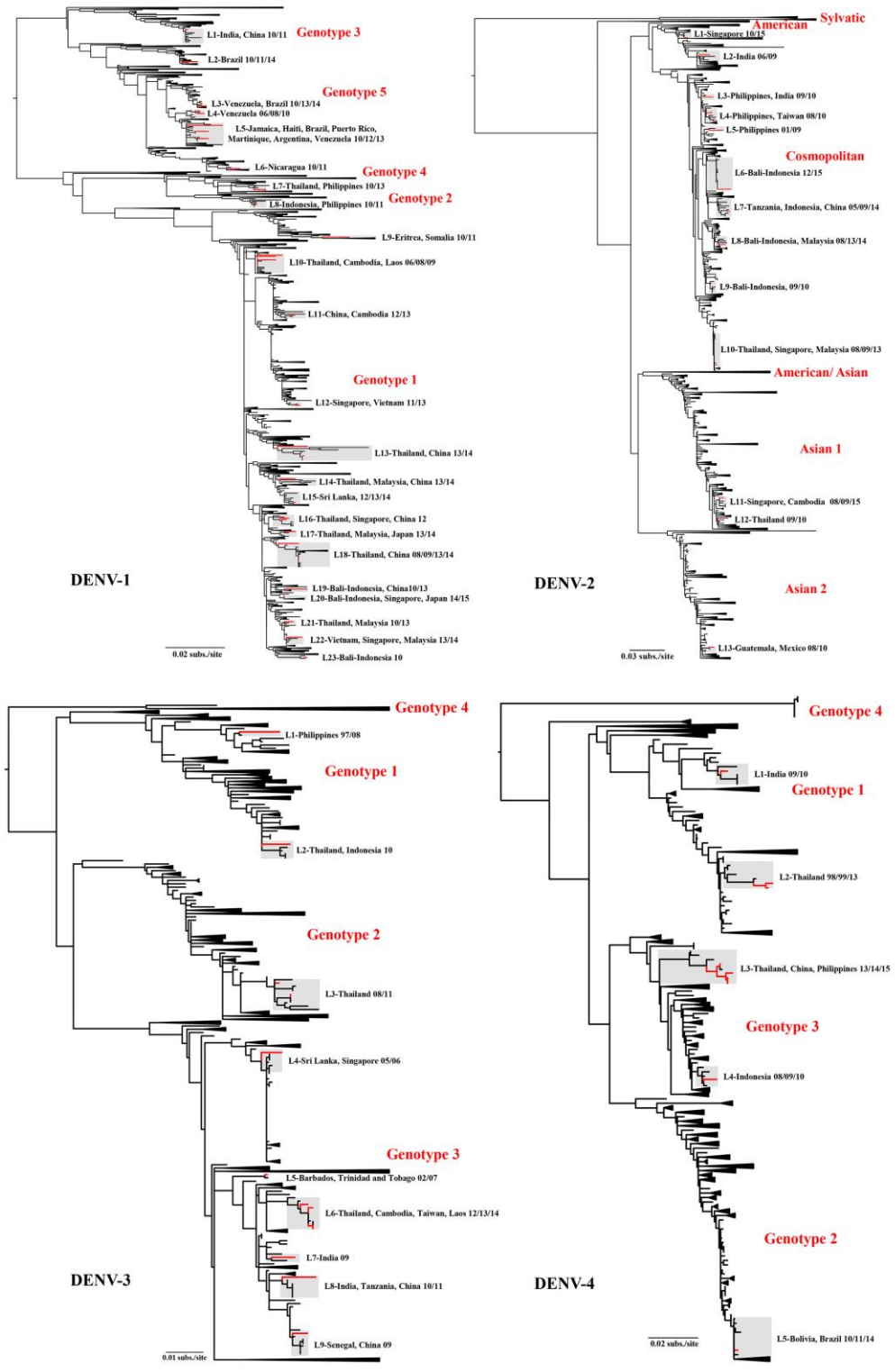
*Nucleotide positions are according to the genomes for DENV-1 (GenBank accession no. KM204119), DENV-2 (GenBank accession no. JX66948), DENV-3 (GenBank accession no. EU482596), and DENV-4 (GenBank accession no. EU854301). DENV, dengue virus; F, forward orientation; R, reverse orientation.

Technical Appendix Table 2. Origin and genetic relatedness of dengue viruses isolated from travelers returning to Germany, 2006–2015*

Sequence ID/GenBank accession no.	Travel history	Year of collection	Type	Genotype/lineage
2522/KU509258	Eritrea	2010	DENV-1	II/L9
2878/KU509259	Ko Samui, Thailand	2008	DENV-1	I/L10
2334/KU509313	Singapore	2013	DENV-1	I/L12
18439/KU509265	NA	2012	DENV-1	I/L11
3616/KU509260	Cambodia	2008	DENV-1	I/L10
18014/KU509263	Thailand	2012	DENV-1	I/L16

Sequence ID/GenBank accession no.	Travel history	Year of collection	Type	Genotype/lineage
18805/KU509266	Thailand	2012	DENV-1	I/L16
8902/KU509289	Bali, Indonesia	2015	DENV-1	I/L23
9328/KU509291	Thailand	2013	DENV-1	I/L21
24278/KU509309	Bali, Indonesia	2013	DENV-1	I/L19
11656/KU509293	Thailand	2014	DENV-1	I/L18
1763/KU509314	Thailand	2013	DENV-1	I/L17
8356/KU509253	Bali, Indonesia	2014	DENV-1	I/L20
11100/KU509292	Vietnam	2014	DENV-1	I/L22
1990/KU509257	Thailand	2008	DENV-1	I/L18
8915/KU509290	Thailand	2013	DENV-1	I/L13
12563/KU509294	Thailand	2014	DENV-1	I/L13
23789/KU509310	Sri Lanka	2013	DENV-1	I/L15
17461/KU509250	Thailand	2012	DENV-1	I/L14
384/KU509256	Thailand	2009	DENV-1	I/L10
3845/KU509262	Thailand	2008	DENV-1	I/L10
00808/KU509316	Thailand	2013	DENV-1	II/L7
1301/KU509315	Philippines	2013	DENV-1	II/L7
3746/KU509261	Bali, Indonesia	2010	DENV-1	II/L8
23444/KU509295	Haiti	2013	DENV-1	III/L5
3852/KU509251	Margarita Island, Venezuela	2008	DENV-1	III/L4
4876/KU509252	Venezuela	2010	DENV-1	III/L4
10429/KU509254	Aragua, Venezuela	2011	DENV-1	III/L3
18037/KU509264	Haiti	2010	DENV-1	III/L5
5353/KU509312	Nicaragua	2014	DENV-1	III/L6
8246/KU509311	Brazil	2014	DENV-1	III/L2
17388/KU509249	Jamaica	2012	DENV-1	III/L5
16687/KU509255	India	2011	DENV-1	III/L1
1365/KU509272	Thailand	2009	DENV-2	Cosmopolitan/L10
3849/KU509275	Philippines	2008	DENV-2	Cosmopolitan/L4
3850/KU509276	Philippines	2008	DENV-2	Cosmopolitan/L4
14706/KU509277	Philippines	2010	DENV-2	Cosmopolitan/L4
3519/KU509274	Philippines	2010	DENV-2	Cosmopolitan/L3
973/KU509269	Philippines	2009	DENV-2	Cosmopolitan/L5
979/KU509270	Bali, Indonesia	2012	DENV-2	Cosmopolitan/L6
4584/KU509308	Bali, Indonesia	2015	DENV-2	Cosmopolitan/L6
6478/KU509307	Tanzania	2014	DENV-2	Cosmopolitan/L7
671/KU509268	Bali, Indonesia	2009	DENV-2	Cosmopolitan/L9
017079/KU509305	Bali, Indonesia	2014	DENV-2	Cosmopolitan/L8
2627/KU509271	India	2006	DENV-2	Cosmopolitan/L2
15766/KU509306	Singapore	2015	DENV-2	Cosmopolitan/L1
3229/KU509273	Thailand	2011	DENV-2	Asian 1/L12
30/KU509267	Guatemala	2010	DENV-2	Asian 2/L13
4397/KX147335	Singapore	2015	DENV-2	Asian 1/L11
254/KU509278	Barbados	2007	DENV-3	III/L5
632/KU509279	Philippines	2008	DENV-3	I/L1
1631/KU509280	Ko Samui, Thailand	2011	DENV-3	II/L3
2994/KU509281	India	2009	DENV-3	III/L7
3140/KU509282	Senegal	2009	DENV-3	III.L9
3404/KU509283	Sri Lanka	2006	DENV-3	III/L4
3847/KU509284	Thailand	2008	DENV-3	II/L3
8561/KU509285	Thailand	2010	DENV-3	I/L2
9468/KU509286	India	2011	DENV-3	III/L8
10803/KU509302	Thailand	2013	DENV-3	III/L6
10407/KU509303	Thailand	2013	DENV-3	III/L6
9846/KU509304	Thailand	2014	DENV-3	III/L6
3274/KU509287	India	2009	DENV-4	I/L1
6120/KU509288	Bali, Indonesia	2010	DENV-4	III/L4
22712/KU509296	Thailand	2013	DENV-4	I/L2
15983/KU509297	Philippines	2015	DENV-4	III/L3
12489/KU509298	Thailand	2015	DENV-4	III/L3
3186/KU509299	Thailand	2013	DENV-4	III/L3
3060/KU509300	Thailand	2013	DENV-4	I/L2
0831/KU509301	Bolivia	2014	DENV-4	II/L5
8635/KX147336	Thailand	2014	DENV-4	III/L3

*The isolates represent cases investigated by the World Health Organization Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research. DENV, dengue virus; NA, not applicable.



Technical Appendix Figure. Phylogenetic relationships, based on complete envelope gene sequences, of dengue virus (DENV) strains isolated from travelers returning to Germany, 2006–2015. For better visualization of the position of the sequences generated during this study, and because of the very large

number of sequences (DENV-1 = 3884 taxa; DENV-2 = 3498 taxa; DENV-3 = 1870 taxa; DENV-4 = 890 taxa) included, several phylogenetic clusters have been collapsed and depicted as triangles. The major genotypes, lineages (gray boxes) in which our sequences (strains sampled from German travelers colored in red) are grouped, and geographic origins of the members of the lineages, as well as the year of isolation, are indicated. Statistical support of grouping (maximum-likelihood bootstrap replicates $\geq$70%) is indicated with an asterisk. Scale bar indicates nucleotide substitution per site.